

Practical Applications of New Laboratory Methods for Plague Investigations *

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A continuing need exists for improved laboratory methods in the diagnosis of plague. Plague investigations in the field, in the diagnostic laboratory or in the research laboratory are generally laborious, time-consuming and expensive, and even under optimum conditions often do not yield all the information sought by the investigator. Delay in diagnosis is a particularly important problem since there is an urgent requirement for quick identification of the plague organism on account of the rapid course and acute nature of the disease and the need for specific therapy to be initiated.

Since the publication of an article on laboratory methods for the diagnosis of plague (Baltazard et al., 1956), investigators, guided by this summary of current knowledge, have gathered useful information in many parts of the world. During this time, several antigenic and other properties of the plague bacillus have been found to be associated with the virulence of this organism (Burrows, 1963). A number of the bacterial characters related to virulence have been of immense practical value as identifying properties in isolating the plague bacillus from clinical material obtained from animals and man. These virulence factors are the V and W virulence antigens (VW), fraction 1 antigen (F1), pigmentation on haemin agar (P), pesticin I (PI), coagulation of plasma (C) and fibrinolytic activity (F); the three latter properties are apparently linked (PI-C-F) (Brubaker, Beesley & Surgalla, 1965).

Detection of virulence factors provides the basis for five newly developed laboratory methods which facilitate identification, isolation and characterization of the plague organism; they are: (1) the antiserum-agar plate technique, (2) pesticin assay agar, (3) magnesium oxalate agar, (4) Congo-red pigmentation agar, and (5) fibrinolytic assay plates. The first

method utilizes fraction 1 antigen and the second utilizes pesticin I as the identifying trait for isolating *Pasteurella pestis* from primary culture plates of specimens. The other three methods provide information on virulence and VW antigen production, pigmentation and fibrinolytic activity, thereby permitting further characterization of the isolates as presumptively virulent, attenuated or avirulent.

In view of the continuing plague problems in Vietnam, recent outbreaks in Nepal and Tanzania, isolations of *P. pestis* in Yemen, and continuing interest in natural foci in western USA, South America, the USSR and other parts of the world, it is felt that a brief consolidated description of these methods may serve as a useful adjunct to the earlier guide (Baltazard et al., 1956). In this paper, the practical value of these methods for examination of clinical specimens is described and illustrated. It is realized that the quality of reagents may vary from place to place; therefore, special details and precautions for the correct preparation of the required materials have been included.

Materials and methods

Antiserum-agar plate technique. Colonies of *P. pestis* can be specifically identified on antiserum-agar plates by a specific fraction 1 antigen-antibody precipitin ring that develops around each fraction-1-positive *P. pestis* colony within 4-6 hours after the colonies are exposed to chloroform vapour (Albizo & Surgalla, 1968a, 1968b).

Antiserum is produced in New Zealand White rabbits (2.5 kg-3 kg) inoculated with plague vaccine.⁴ Initially, 0.75 ml of an equal mixture of vaccine and Freund's complete adjuvant (Difco) is injected intramuscularly into each hind leg and 0.5 ml is injected subcutaneously in the dorsal neck region. One week later, 0.5 ml of undiluted vaccine without adjuvant is injected intramuscularly into one hind leg and 0.5 ml is injected subcutaneously in the dorsal neck region. The rabbits yield high-titre fraction 1 antibody from about the 9th week to the end of the

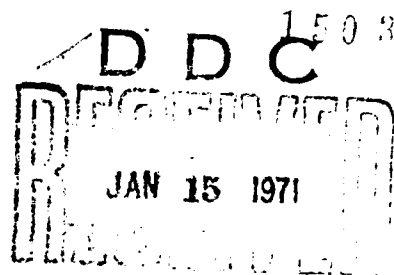
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⁴ Produced by Cutter Laboratories, Berkeley, Calif., USA.



16th week (Albizo & Surgalla, 1968a). The antisera (weekly pools) can be rendered specific for *P. pestis* simply by diluting out unwanted antibodies that are present at low titre.

Filtered antiserum at a predetermined dilution is mixed with an equal volume of sterile 8% blood-agar base (Difco; pH 6.8); 10-ml quantities are poured into standard Petri plates and the solidified agar plates are stored at 4°C in sealed plastic bags until used. Media other than blood-agar base, such as a deoxycholate-copper sulfate selective medium (Albizo & Surgalla, 1968b), may be used provided they are previously tested for effectiveness, since some media allow the formation of more distinct rings than others do. Inoculated plates are incubated at 37°C for 40–66 hours or until colonies are 0.5 mm–1.0 mm in diameter. Plates are then exposed to chloroform vapour and incubated again at 37°C. After a further 4–6 hours colonies are observed for precipitin rings formed around colonies. In order to observe the rings clearly, a source of oblique transmitted light, a dark background, and some magnification, such as that provided by a dissecting microscope, are required.

Special considerations: (1) antiserum must previously be tested for F1 specificity at the dilution selected for the actual test; (2) the agar medium must previously be tested for effectiveness; (3) colonies 1 mm in diameter should not be exposed for longer than 60 seconds to chloroform vapour if a viable isolate from the colony is desired.

Pesticin assay agar. The bacteriocin, pesticin I (Ben-Gurion & Hertman, 1958), diffusing from *P. pestis* colonies inhibits the growth of *P. pseudotuberculosis* in a seeded agar overlay. An efficient assay medium was devised by Brubaker & Surgalla (1961, 1962). The medium is prepared as follows:

Components and procedure	Quantity
Blood-agar base *	4%
Sterilize at 121°C for 15 minutes, then cool to 45°C–50°C and add:	
Calcium chloride (sterile solution)	0.01 M final concentration
Sodium calcium edetate	0.1% final concentration
Glucose	0.1 M final concentration

* Supplied by Baltimore Biological Laboratories, Baltimore, Md., USA.

Pesticin I is genetically linked to fibrinolytic and coagulase activities and these properties are related to virulence (Brubaker, Beesley & Surgalla, 1965).

Identification and isolation from primary cultures on this medium are greatly facilitated (Beesley & Surgalla, 1969).

The base layer is poured and dried at room temperature; it is then inoculated and incubated for 48 hours at 26°C. Colonies are exposed to chloroform vapour by inverting the plate over a chloroform-saturated gauze pad (about 5 cm by 5 cm). The vapour is then allowed to dissipate for 1 minute. Melted pesticin agar at 45°C is seeded with type I *P. pseudotuberculosis* (10^5 organisms/ml); 5 ml of seeded overlay is then pipetted on to the culture of plague bacilli. The culture is incubated at 37°C for 18–24 hours, then observed for clear zones of inhibition around *P. pestis* colonies.

Special considerations: (1) Optimum results are obtained with 10^5 – 10^6 indicator organisms per ml of agar. (2) Initial incubation may be carried out at 26°C or 37°C; however, incubation after overlay with the indicator culture must be carried out at 37°C. (3) A specimen may be recovered from the centre of the zone; restreaking on a medium such as Congo-red agar permits easy differentiation of the plague bacilli from the indicator strain, *P. pseudotuberculosis* type I. (4) Chloroform treatment in excess of 1 minute decreases the possibility of recovering viable organisms.

Magnesium oxalate agar. This medium, devised by Higuchi & Smith (1961), inhibits growth of VW+ *P. pestis* but allows VW- organisms to form colonies normally. The original experiments relating this nutritional peculiarity to VW antigens and to virulence have been summarized by Surgalla, Andrews & Cavanaugh (1968).

The medium is prepared as follows:

Components and procedure	Quantity
Blood-agar base	40 g
Distilled water	830 ml
Sterilize at 121°C for 15 minutes, then cool to 45°C–50°C and add aseptically:	
Magnesium chloride	80 ml/0.25 M
Sodium oxalate	80 ml/0.25 M
Glucose	10 ml/1.0 M

Special considerations: (1) Media containing low concentrations of calcium must be used. It has been found that blood-agar base supplied by Baltimore Biological Laboratories is very satisfactory. (2) Incubation must be carried out at 37°C for 48–72 hours. (3) Dilutions containing between 5×10^3 and 5×10^4 organisms per 0.1 ml should be used to inoculate the surface of duplicate plates. One plate should be

FIG. 1-7

APPLICATIONS OF THE FIVE LABORATORY METHODS FOR THE INVESTIGATION OF PLAGUE



- Fig. 1: *P. pestis* colonies identified by precipitin rings in deoxycholate antiserum-agar plates containing copper sulfate. Plate inoculated with homogenized human bone specimen and incubated at 37 C.
- Fig. 2: Control blood-agar base plate inoculated with the same material as in Fig. 1; incubated at 37 C.
- Fig. 3: Pesticin assay agar plate inoculated with 2×10^{-7} g of bone marrow specimen. *P. pestis* colonies are identified by a clear zone of inhibition; incubation at 37 C.
- Fig. 4: Congo-red pigmentation agar plate differentiating a mixture of pigmented and non-pigmented variants of *P. pestis* (strain Kim-10); incubated at 26 C.
- Fig. 5: Bovine fibrin plate demonstrating 5 positive and 3 negative tests for fibrinolysis; incubated at 37 C for 24 hours.
- Fig. 6 and 7. Magnesium oxalate agar plates inoculated with 2.8×10^5 *P. pestis* (strain Alexander). Plates in Fig. 6 and 7 were incubated at 26 C and 37 C, respectively. Growth at 26 C is virulent and VW⁺ whereas colonies appearing at 37 C are avirulent VW⁻ variants.

incubated at 37°C and the other, used as a control, is incubated at 26°C–28°C. A "calcium dependent" (VW+) culture will produce confluent growth at 26°C and 100 colonies or less at 37°C.

Congo-red pigmentation agar (Surgalla & Beesley, 1969a). Red colonies are produced on this agar by *P. pestis* which would produce dark-brown colonies on the defined haemin agar of Jackson & Burrows (1956); non-pigmented colonies are produced by organisms which form non-pigmented colonies on haemin agar.

The medium is prepared as follows:

Components and procedure	Quantity
Heart-infusion broth	10 g
Bacto agar	20 g
Galactose	2 g
Congo-red stain	0.1 g
Distilled water	1 litre
Sterilize at 121°C for 15 minutes	

Special considerations: (1) Heart-infusion broth supplied by Difco, Detroit, Mich., USA, and infusion broth supplied by Baltimore Biological Laboratories, Baltimore, Md., USA, are very satisfactory media. (2) Incubation must be carried out at 26°C–28°C. (3) Some strains of *P. pestis* do not pigment as quickly or intensely as others. (4) EV 76 is a useful non-pigmented standard strain and avirulent VW- pigmented strains, such as Tjiwidej, provide adequate controls. (5) Galactose is the only tested carbohydrate that may be used in the medium.

Fibrinolytic assay plates. Plague fibrinolytic factor activates plasminogen to plasmin, resulting in a proteolytic dissolution of fibrin seen as a clear liquid spot on an opalescent fibrin film (Beesley, Brubaker & Surgalla, 1967; Beesley, Janssen & Surgalla, 1963). The bovine plate method was devised by Astrup & Mullertz (1952), the *P. pestis* fibrinolytic factor having been discovered by Madison (1936). Burrows (1968) used this procedure with growth media for the easy identification of individual colonies as fibrinolytic or non-fibrinolytic.

The medium is prepared as follows: 10 ml of a 0.25% solution of fibrinogen are placed in a Petri dish. Sodium borate buffer (0.5 ml; pH 7.75), prepared by the method of Lewis & Ferguson (1950) and containing 25 US National Institutes of Health units of bovine thrombin, is added with gentle swirling. Coagulation occurs in 20–60 seconds. Cultures are spotted on the surface of the medium and incubated at 37°C.

Special considerations: (1) Bovine fibrinogen supplied by Armour, Chicago, Ill., USA, and bovine thrombin topical supplied by Parke-Davis, Detroit, Mich., USA, are very satisfactory. Other preparations may be adapted if satisfactory results are obtained with control organisms. (2) Fibrinogen must be filtered to eliminate proteolytic, spore-forming organisms. (3) Thorough mixing of thrombin with fibrinogen is required to prevent soft areas in the film. (4) Incubation may be carried out between 26°C and 37°C; however, lysis occurs much faster at the higher temperature.

Applications of the methods

Applications of the 5 laboratory methods are illustrated in Fig. 1–7. The effectiveness of antiserum agar and pesticin assay agar for the isolation of *P. pestis* from a contaminated human bone specimen (Nepal 20a, 1967; provided by Dr P. S. Brachman, US Public Health Service) is demonstrated in Fig. 1–3. Deoxycholate agar containing specific fraction 1 antiserum plus 0.025% copper sulfate (Fig. 1) and blood-agar base (Fig. 2) were inoculated with equal amounts of bone homogenate. The selective antiserum-agar inhibits the growth of many contaminants that grow on blood-agar base and readily permits the identification of *P. pestis* colonies by the presence of surrounding specific precipitin rings. Fig. 3 illustrates 3 easily identifiable *P. pestis* colonies on pesticin assay agar inoculated with approximately 2×10^{-7} g of bone marrow. The *P. pestis* colonies are surrounded by clear zones of inhibition.

Fig. 4 illustrates pigmented and non-pigmented colonies produced on Congo-red pigmentation agar inoculated with a mixture of the pigmented strain Kim-10 with a non-pigmented variant of this strain. Fibrinolytic activity illustrated in Fig. 5 shows a bovine fibrin film spotted with 5 fibrinolytic and 3 non-fibrinolytic cultures. The eight cultures are (clockwise from the top): (1) Kim-10, positive control; (2) Nepal 20a; (3) Garcia, New Mexico 1969; (4) Saganey, Arizona 1968; (5) G32, negative control; (6) Bolivia, 1969; (7) *P. pseudotuberculosis* type I strain PB1/+; (8) *Yersinia enterocolitica* Winblad. The use of magnesium oxalate agar to determine the ability of isolates to produce VW antigens is shown in Fig. 6 and 7. The two magnesium oxalate agar plates were incubated with equal amounts of virulent *P. pestis* strain Alexander. No inhibition of growth is seen on one plate incubated at 26°C (Fig. 6). However, on the other plate, incubated at 37°C

(Fig. 7), growth of virulent organisms is inhibited and only avirulent VW⁻ variants are able to produce colonies.

Discussion

Recent studies suggest that the ability of the plague bacillus to establish an infection in animals inoculated with small numbers of organisms appears to be dependent on VW⁺; however, a lethal outcome of infection appears generally to require F1⁺, P⁺ and PI⁺-C⁺-F⁺ (Surgalla & Beesley, 1969b; Janssen & Surgalla, 1969; Donovan et al., 1961). Variants negative for VW, P, F1, or PI-C-F can be readily selected from virulent plague cultures in the laboratory. Attenuated strains recently isolated from nature include the American human isolates Bryans (Winter, Cherry & Moody, 1960) and Dodson (Surgalla & Beesley, 1969b), strain 1260 representing the vole variety from Armenia (Levi et al., 1961), the Petit Caucase, Altai and Transbaikalie varieties described by Martinevskij (1968) and other rodent strains from the western USA (Marchette, 1963; Thorpe, Marchette & Bushman, 1963; Rust & Cavanaugh, 1964). Certain stock cultures of the classical P⁻ strain EV 76 have been noted by us and by J. D. Marshall (personal communication) to contain large populations of PI⁻ organisms, and animal experimentation with such cultures can yield atypical results. We may expect, therefore, that attenuated variants of the plague bacillus are to be found in natural foci throughout the world and an

understanding of their role in the ecology of plague will require a greater effort being made to identify and isolate attenuated, as well as virulent, plague organisms.

As a supplement to current methodology, including the fluorescent antibody staining technique (Winter & Moody, 1959), the recently developed methods described in this note have several special advantages. They are particularly useful in the absence of laboratory animals and with minimally equipped laboratories, and they can be used in various combinations according to the immediate needs and capabilities of the investigator. For the antiserum-agar plate technique, any laboratory should be able to produce specific F1 antiserum with commercially available materials, Cutter plague vaccine, Freund's adjuvant, and rabbits. Pesticin assay agar utilizes simple and inexpensive components and is amenable to use in any laboratory. By simply observing growth on magnesium oxalate agar plates, reliable information may be obtained regarding virulence and production of the V and W antigens which Burrows (1963) discovered to be essential for full virulence in *P. pestis* and *P. pseudotuberculosis*. Congo-red pigmentation agar appears to give the same information as haemin agar in regard to virulence attenuation and is easier and less time-consuming to prepare. Isolates may be identified as fibrinolytic within 3 hours on fibrinolytic assay plates (J. D. Marshall, personal communication). Each of the methods thus supplies presumptive information relating to virulence without the use of animals.

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